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Kv1.1 channel antisense attenuates learning and modulation of dentate polysialylated NCAM

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THE distribution and modulation of neural cell adhesion molecule polysialylation state (NCAM PSA) and the consequence of antisense inactivation of the Kv1.1 potassium channel was investigated following avoidance learning in mice. PSA immunoreactivity was most notable on cells at the inner denate border and in cortical layer II. Task acquisition resulted in a significant 30% transient increase in the frequency of dentate polysialylated neurons at the 1 2 h post-training time. In contrast, animals pretreated with the Kv1.1 antisense oligonucleotide exhibited both attenuated recall avoidance latencies and polysialylated cell frequency. As Kv1.1 is enriched on the dendrites of these granule-like cells, the attenuated polysialylation response is considered secondary to NCAM-mediated events during their transient synapse production in the 6– 8 h post-training period. *NeuroReport* 9: 2727–2731 © 1998 Lippincott Williams & Wilkins.

Key words: Granule cells; Hippocampal formation; Neuroplasticity; Passive avoidance; Voltage-gated potassium channels

Introduction

Long-term memory storage has been proposed to involve the translation of activity-dependent signals into a cellular cascade of gene expression, altered protein synthesis and the growth of new synaptic connections mediated, in part, by glycoproteins involved in the adhesion of neural cells.[1,2] In the adult rat, the neural cell adhesion molecule (NCAM) is required in the 6–8 h post-training time. This immediately follows a period of learning-dependent protein synthesis and is coincident with transient, learning-associated increases in hippocampal dentate granule cell spine density.[3] Subsequently, in the 10–12 h post-training period, NCAM is transiently glycosylated with homopolymers of α2,8-linked polysialic acid (PSA), a major post-translational modification observed at neuroplastic sites undergoing synaptic rearrangement within the adult central nervous system.[4,5] Transient, learning-induced change in the frequency of polysialylated neurons is not restricted to the hippocampal dentate gyrus as similar modulations have been observed to occur in a bidirectional cortico-hippocampal pathway involving the entorhinal, perirhinal and piriform cortex.[6] These transient changes are specific to learning as they fail to occur in animals rendered amnesic with scopolamine or apomorphine and are preserved by co-administration of an anti-amnesic agent.[7]

Although learning-associated modulations of NCAM polysialylation state are specific to both spatial and non-spatial forms of learning in the adult Wistar rat,[4] it is uncertain whether these are common to other rodents and to what extent variations in conditioning tasks influence the extent of these neuroplastic events in memory consolidation. To address these issues, we have mapped the distribution of NCAM polysialylation in the mouse cortico-hippocampal pathway and determined whether transient modulations in the frequency of dentate polysialylated neurons occurred following training in a variant of the passive avoidance paradigm employed in previous studies. In addition, as the association of amnesia with loss of learning-associated modulations in NCAM polysialylation state has employed agents with limited regional specificity, such as scopolamine,[7] we employed an amnesic agent with relatively specific effects on the cortico-hippocampal pathway in which these learning-associated neuroplastic events occur. Recently, the Kv1.1 late rectifying potassium channel has been localized to dendrites both in hippocampal CA3 and dentate granular cells and shown to be essential for both spatial and non-spatial forms of learning.[8–10] We therefore employed an antisense oligonucleotide strategy to inactivate the Kv1.1 potassium channel and determine the consequence of its amnesic action on the learning associated modulations of dentate NCAM polysialylation state.

Materials and Methods

Animals: Postnatal day 65 Male Swiss albino mice (23–30 g) from Morini (San Polo d'Enza, Italy) were used. Fifteen mice were housed in each cage and maintained at 23 ± 1°C on a 12:12 h light:dark cycle (lights on at 07.00 h). Postnatal day 80 male Wistar rats (300–350 g), obtained from the Biomedical Facility, University College Dublin, were maintained in a similar manner.

Passive avoidance paradigm: A step-through passive avoidance paradigm was employed as described previously.[10] Briefly, the apparatus consisted of light and dark compartments separated by a guillotine door. The latter compartment contained a pitfall floor with access to a cold water bath (10°C). Upon entering the darkened chamber with all four paws, the animal was dropped into the water bath. Latency times (s) to enter the darkened chamber were measured during training and at 12 h post-training recall time and used as an indication of successful learning of the paradigm. All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by National Institute of Health and the experimental protocol was approved by the Ethical Committe of the Department of Pharmacology, University of Florence. Non-parametric statistical comparisons were made initially using the Kruskal-Wallis analysis of variance (ANOVA) followed by the Mann-Whitney U-test and $p < 0.05$ was considered to be significant.

Immunohistochemistry: Animals trained in the avoidance task were sacrificed immediately or at 12 h post-training. Their brains were removed rapidly, coated in optimal cutting temperature compound (Agar Scientific Ltd, UK), snap-frozen in dry ice-cooled *n*-hexane and stored at –80°C until required for further processing. PSA immunocytochemistry was employed to determine the distribution of hippocampal NCAM PSA, using a monoclonal antibody specific for NCAM PSA as described previously.[11] Briefly, cryostat axial sections (12 μm) were fixed in 70% (v/v) ethanol and incubated overnight with anti-PSA ascitic fluid diluted 1:500 (generous gift of Professor G. Rougon). The sections were exposed for 3 h to FITC-conjugated goat anti-mouse IgM diluted 1:100 (Calbiochem, UK) and mounted in Citifluor (Agar, UK), a fluorescence-enhancing medium. Immunofluorescence is specific as previous studies have demonstrated it to be eliminated completely by prior incubation of the sections with 0.3% v/v endoneuraminidase-N.[4] Nuclei were fluorescently counter-stained by a brief exposure (60 s) to propidium iodide (40 ng/ml PBS; Sigma Chemical Co., UK) to facilitate counting.

The frequency of PSA-immunopositive neurons in the granule cell layer and hilar border was determined by direct counting in seven alternate 12 μm sections commencing at Bregma level –3.25 mm. Cell counts were divided by the total area of the granule cell layer and multiplied by the average granular cell layer area (0.15 ± 0.01 mm^2 at this level). The mean was calculated for each animal and data are expressed as mean ± s.e.m. number of PSA-positive cells per unit area for each experimental group. Area measurements were performed using a Quantimet 500 Image Analysis System. Statistical comparisons were made initially using an ANOVA followed by the one-tailed Student's *t*-test, with $p < 0.05$ considered significant.

Kv1.1 potassium channel inactivation: The Kv1.1 potassium channel was inactivated by intracerebroventricular (i.c.v.) injections of 24mer phosphodiester oligonucleotides (ODN), capped by a terminal phosphorothioate double substitution and purified by chromatography (Genosys, The Woodlands, USA). The antisense oligodeoxyribonucleotide (5′-CGACATCACCGTCATGATGAAAGC-3′) was designed to target the 5′ portion of the murine Kv1.1 (mKv1.1) mRNA at residues 575–598 of the published cDNA sequence.[12] The antisense ODN employed was specific for Kv1.1 as previous scanning of the GenBank database (accession number M30439) against cDNA sequences revealed low homology with other members of the voltage-dependent potassium channel superfamily.[12] A fully degenerated 24mer oligodeoxyribonucleotide was used as control. The oligodeoxyribonucleotides were preincubated at 37°C for 30 min with 13 μM DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulfate), which also served as a vector.

The mice were randomly assigned to one of four groups: non-injected, antisense ODN injected, degenerated ODN injected and vector injected. The ODNs (1 nmol) and vector were administered once every 72 h, on three successive occasions, and the animals were trained 48 h after the last injection. The injections were made by the i.c.v. route under ether anesthesia using isotonic saline as a solvent.[10] Briefly, during anesthesia a hypodermic needle (0.4 mm o.d.) attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain, where 5 μl was then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through the anterior base of the ears. Injections were performed into the left and right ventricles in a random fashion. To ascertain that the ODNs were administered exactly into the lateral ventricle, some mice were injected with

5 μl 1:10 diluted India ink and their brains examined macroscopically after sectioning to determine dye distribution. Those inappropriately injected were excluded from the study.

Results and Discussion

Expression of NCAM polysialylation in the mouse medial temporal lobe: At Bregma level –3.25 mm, PSA immunoreactivity within the mouse hippocampal formation was most notable in the hilus and dentate gyrus (Fig. 1). This diffuse staining continued along the mossy fiber path and included the CA3 and CA2 regions. In contrast, PSA expression in the fimbria, pyramidal cell layer, stratum radiatum and regions containing fibers returning from the CA1 region to the subiculum was relatively weak compared with that observed in the subiculum, lacunar molecular layer and dorsolateral geniculate nucleus. A similar distribution of PSA immunoreactivity has been described previously in the mouse, rat and human, using the same monoclonal antibody[6,13] and similar antibodies directed against neuraminidase-sensitive NCAM epitopes (12F8[14] and 12E3[15,16]). Additionally, NCAM PSA expression was dominant in layers I and III of the entorhinal cortex and, in its more ventrolateral region, continued to be expressed as a single band of neurons in layer II. This band of immunopositive cells extended anteriorly to include the perirhinal and piriform cortices at Bregma levels –4.75 and –5.0 mm (Fig. 1). This confirms previous studies in the rat which have identified the presence of a band of NCAM PSA-positive cells extending from the entorhinal cortex, through the perirhinal cortex and into the piriform cortex.[6,13,17] Given that NCAM PSA expression has been consistently associated with morphofunctional change,[5] it must be assumed that across all species a neuroplastic pathway is retained in the adult hippocampal formation.

Learning-associated modulations of NCAM polysialylation: Within the dentate gyrus of the mouse hippocampal formation a population of PSA-immunopositive cells exhibited intense fluorescence (Fig. 1), as has been observed previously in the hippocampal formation of the rat and human.[4,13–16] These are located at the border of the granule cell layer and hilus and extend numerous dendrites into the molecular layer, the inner third of which exhibits diffuse immunostaining. In the mouse, the frequency of these polysialylated neurons was highest at Bregma level –3.0 to –3.25 mm compared with –5.6 mm in the rat (Fig. 2), at which the gross morphology of the dentate was comparable in both species. In contrast, the polysialylated neurons of the mouse

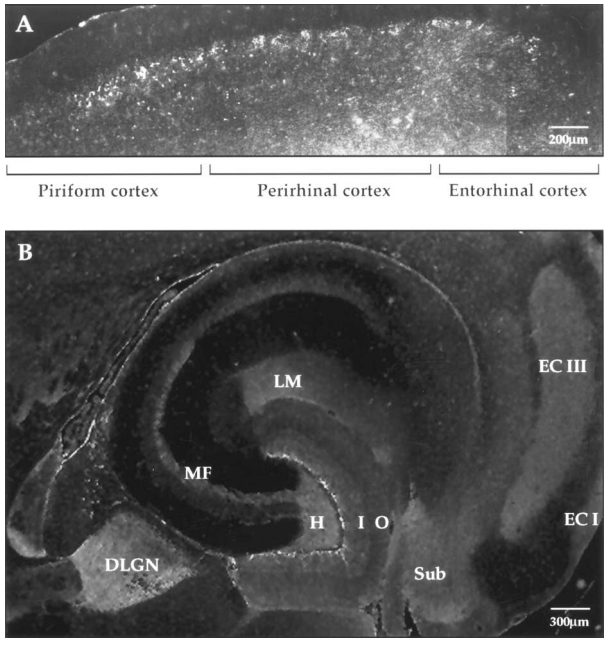

FIG. 1. Expression of PSA immunoreactivity in the murine cortico-hippocampal pathway. (**A**) Montage of photomicrographs illustrating a discrete band of PSA-immunopositive neurons within layer II of the entorhinal/perirhinal/piriform cortices at 4.75 mm below Bregma (×55). (**B**) Photomontage showing relative PSA immunostaining intensity in the hippocampal formation and associated entorhinal cortex at Bregma level –3.25 mm (×50). H: hilus; I: inner molecular layer; O: outer molecular layer; Sub: subiculum; MF: mossy fibres; ECI: layer I of the entorhinal cortex; ECIII: layer III of the entorhinal cortex; LM: lacunar molecular layer; DLGN: dorsolateral geniculate nucleus.

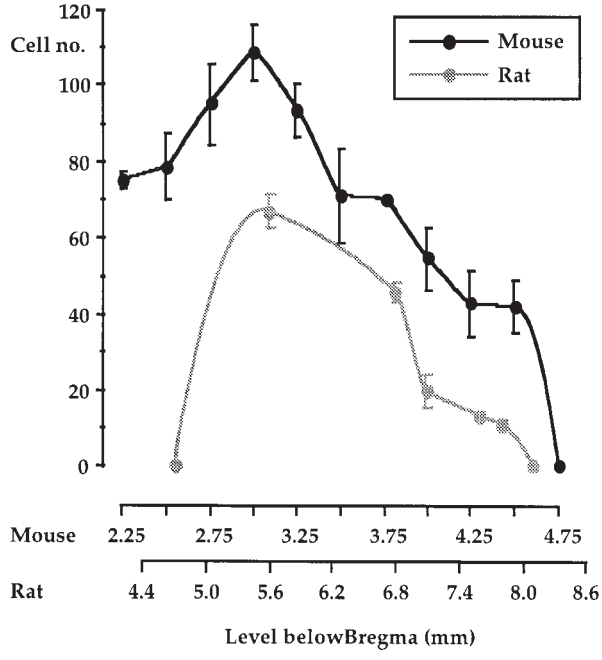

FIG. 2. Correlation of hippocampal polysialylated neuron frequency at the dentate granule cell layer and hilar border with descending levels from Bregma in the rat and mouse. Data are mean ± s.e.m. (n = 3–6).

were clustered in a more dorso-medial aspect compared with their dominant medial location in the rat. This is unlikely to be an age-related phenomenon, as based on average life expectancy, both species were sacrificed at an equivalent postnatal period. Moreover, the basal frequency of these polysialylated neurons at Bregma –3.25 mm in the mouse was higher than that observed at –5.6 mm in the rat (86.2 ± 7.0 *vs* 64.4 ± 3.9, respectively).

Previous studies have implicated these PSA-immunopositive cells in the process of ongoing neurogenesis in the rat dentate gyrus;[16] however, only a small percentage of these cells can be labeled with 5-bromo-2′-deoxyuridine.[18] Given their remarkable heterogeneity with respect to their morphology, dendritic projections and electrophysiological response,[19,20] it is unlikely that NCAM PSA is present exclusively on newborn cells, but rather that it is associated with other neuroplastic functions including long-term memory consolidation. In the rat, their frequency increases by ≈60% in the 10–12 h following training in a passive avoidance paradigm and a similar change occurs following spatial forms of learning.[4] In the mouse, a smaller increase of 30% was observed following training in the present variant of the passive avoidance task. Animals readily learned the task as they exhibited a marked increase in avoidance latency upon re-exposure to the training apparatus 12 h post-training (97.3 ± 15 *vs* 28.9 ± 8.7 s at recall and training, respectively). This resulted in a coincident increase in the frequency of dentate polysialylated neurons at 12 h post-training, which was significant with respect to that observed in naive animals or those sacrificed immediately after training ($F = 4.81$, $p = 0.026$; Fig. 3). Moreover, the eventual learning increase in PSA cell frequency in both the mouse and rat was the same (112.0 ± 6 *vs* 101.2 ± 5.4, respectively) suggesting that the response can become saturated. Thus learning-associated modulations in the frequency of polysialylated dentate neurons is a common feature and occurs to the same extent in all species of rodents examined to date.

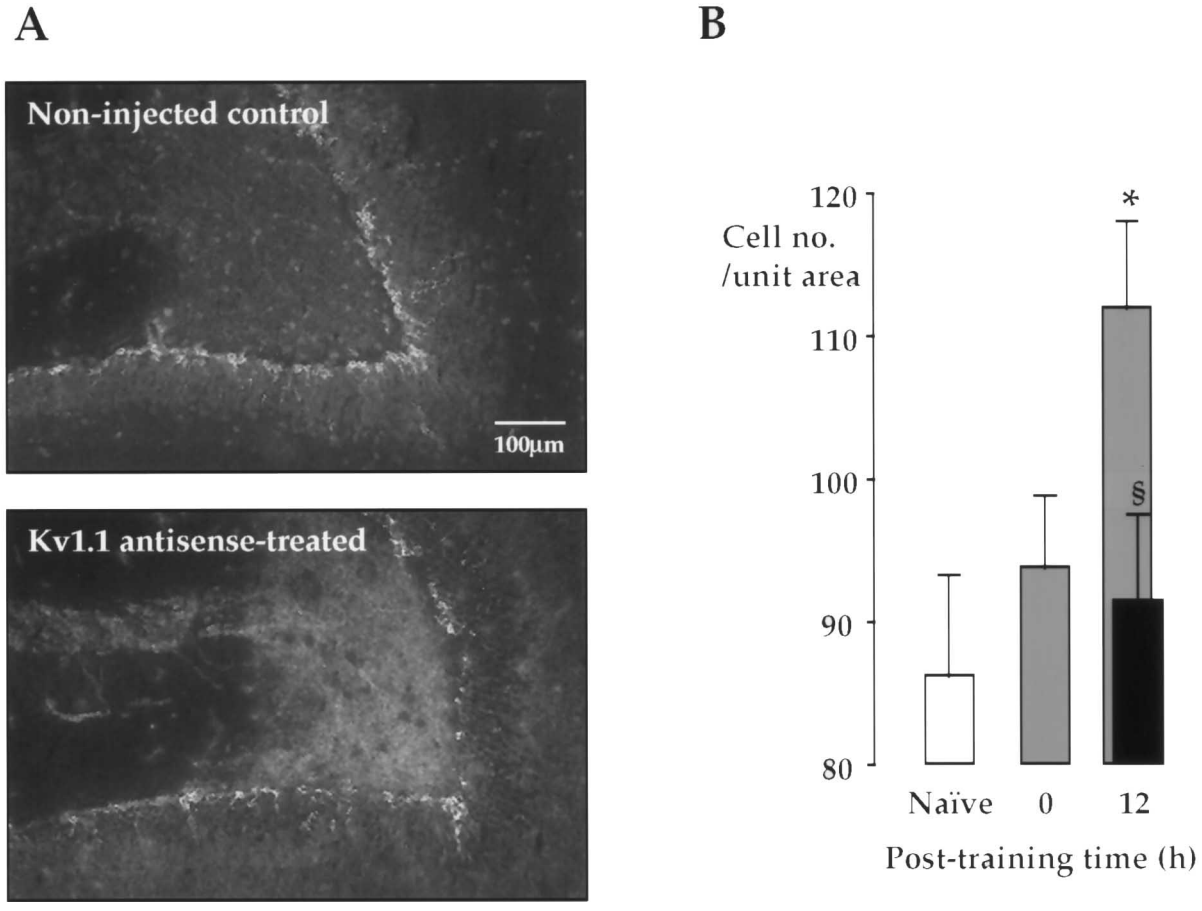

FIG. 3. Learning-induced modulation of dentate NCAM polysialylation in control and Kv1.1 antisense-treated animals. The photomicrographs in (**A**) are qualitative representations of polysialylated cell frequency at the dentate granule cell layer/hilar border 12 h following training in the passive avoidance paradigm in non-injected control and antisense-treated animals (×200). The histogram in (**B**) represents quantitative estimates of polysialylated cell frequency in non-injected control (stippled columns) and Kv1.1 antisense-treated animals (filled column). All values are mean ± s.e.m. $n = 4$–6) and values significantly different ($p < 0.05$) from naive and 12 h post-training non-injected animals are indicated by * and §, respectively.

Behavioural and NCAM PSA deficits induced by Kv1.1 channel antisense: To determine whether these learning-associated modulations of dentate NCAM PSA were specific to learning, animals were treated with aODN directed to the Kv1.1 voltage-gated potassium channel as its appropriate functioning is essential for non-spatial forms of learning such as the passive avoidance paradigm employed in this study.[10] Animals pretreated with the Kv1.1 aODN exhibited recall avoidance latencies that were significantly lower than those observed in animals which had received the dODN or vector ($KW = 10.51$, $p = 0.015$; Table 1). Antisense ODN treatment also resulted in an attenuation of the learning-associated modulations of NCAM PSA as polysialylated dentate cell frequency was significantly decreased with respect to the non-treated controls 12 h after training ($F = 3.2$, $p = 0.047$) and indistinguishable from that observed in the naive animal or those sacrificed immediately following training (0 h; Table 1).

These results confirm and extend previous findings implicating the role of voltage-gated potassium channels in invertebrate and vertebrate models of memory acquisition and consolidation.[10,21,22] The amnesic effects obtained by aODN inactivation of the Kv1.1 potassium channel appear to be specific as K^+ channel agonists in general produce amnesia of a passive avoidance response, an effect reversed by co-administration of K^+ antagonists.[23] In this regard, antisense attenuation of the Kv1.1 channel function is probably specific to the hippocampal dentate granule cell dendrites which mediate fast forward inhibition during learning.[8,9,24] Moreover, as antibody activation of NCAM in cultured cells dampens both A-type and delayed rectifier K^+ currents,[25] the role of this adhesion molecule would be attenuated during granule cell synaptic elaboration at 6–8 h post-training and thereby obviate the need of a polysialylation response at 10–12 h post-training.[4] However, as the lack of significant difference between polysialylated cell frequency, but not recall, in trained animals which received aODN and dODN separately cannot be explained by increased variance in the data (Table 1), the nature of this apparently specific effect on NCAM polysialylation is unclear.

Table 1. Influence of Kv1.1 potassium channel antisense on passive avoidance recall and the activation of dentate polysialylation at the 12h post-training time

	Passive avoidance recall latency (sec)	Polysialylated cell number
Non injected animals		
Naïve	–	86.26 ± 7.0
0h post-training	–	93.86 ± 9.0
12h post-training	102.6 ± 38.4	112.0 ± 6.0*
Antisense-injected animals		
Naïve	–	89.2 ± 9.0
0h post-training	–	87.8 ± 8.0
12h post-training	35.0 ± 21.1§	91.5 ± 6.0§
Degenerative antisense-injected animals	118.7 ± 45.2	106.0 ± 7.0*
Vehicle-injected animals	101.5 ± 51.3	112.0 ± 4.0*

Values are the mean ± SEM ($4 < n < 6$) and number of polysialylated neurons are expressed per unit dentate area.
* $p < 0.05$ vs. non-injected naive animals
§ $p < 0.05$ vs. non-injected animals 12h post-passive avoidance training

Conclusion

Modulations of NCAM polysialylation state associated with synaptic elaboration in the 10–12 h post-training period of memory consolidation are now demonstrated to occur in the murine hippocampal dentate gyrus following passive avoidance training in a manner described previously for the rat. Moreover, antisense inactivation of the Kv1.1 potassium channel, which is enriched on the dendrites of these granule-like cells, produces amnesia for this task and an attenuated modulation of NCAM polysialylation state, potentially by influencing NCAM-mediated events during transient synapse production by granule cells in the 6–8 h post-training.

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